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RESEARCH PAPER

Tissue-specific *PhBPBT* expression is differentially regulated in response to endogenous ethylene

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Abstract

Ethylene is a gaseous plant hormone involved in many physiological processes including senescence, fruit ripening, and defence. Here the effects of pollination and wound-induced ethylene signals on transcript accumulation of *benzoyl CoA:benzyl alcohol/ phenylethanol benzoyltransferase* (*PhBPBT*) are shown in *Petunia×hybrida* cv. Mitchell 'Diploid' (MD). In petunia, *PhBPBT* is responsible for the biosynthesis of both benzyl benzoate and phenylethyl benzoate from benzyl alcohol and phenylethanol, respectively. RNAi-silenced lines, with reduced *PhBPBT* transcript, displayed reduced benzyl benzoate emission, and increased benzyl alcohol levels. Detailed expression analysis showed that *PhBPBT* is regulated by both light and an endogenous circadian rhythm, while it is also differentially regulated in response to ethylene in a tissue-specific manner. Twenty-four hours following pollination of MD flowers, expression of *PhBPBT* decreases in the corolla, while it increases in the ovary after 48 h. This is caused by ethylene that is emitted from the flower coinciding with fertilization as this is not observed in transgenic ethylene-insensitive plants (*CaMV35S::etr1-1*; 44568). Ethylene is also emitted from vegetative tissue of petunia following mechanical wounding, resulting in an increase in *PhBPBT* expression in the leaves where expression is normally below detection levels. Indicative of this pattern of expression, we hypothesize that PhBPBT and subsequent benzyl benzoate production is involved in defence-related processes in the corolla prior to pollination, in the ovary immediately following fertilization, and in vegetative tissue in response to wounding.

Key words: Benzenoids, benzyl benzoate, ethylene, floral volatiles, *Petunia hybrida*, wounding.

Introduction

Floral scent is composed of a wide variety of volatile organic compounds emitted at ratios that are unique to individual species. They can be divided into four major classes depending on their structure: fatty-acid derivatives, benzenoids and phenylpropanoids, terpenoids, and nitrogen-containing or sulphur-containing compounds (Knudsen *et al*., 1993; Schuurink *et al*., 2006). *Petunia×hybrida* cv. 'Mitchell Diploid' (MD) has become an excellent model system for the study of floral volatile synthesis because of a well-established transformation protocol (Jorgensen *et al*., 1996), short life cycle, strong floral aroma, the availability of ethylene-insensitive transgenic plants (35S::*etr1-1*) (Wilkinson *et al*., 1997), and a collection of more than 10 000 ESTs made from randomly sequenced flower cDNA libraries (Underwood, 2003). MD floral aroma is primarily composed of 13 benzenoid/ phenylpropanoid compounds. Using GC–MS analysis these compounds were identified as benzaldehyde, benzyl alcohol, phenylacetaldehyde, benzyl acetate, methyl benzoate, 2-phenylethanol, methyl salicylate, 2-phenylethyl acetate, vanillin, eugenol, isoeugenol, benzyl benzoate, and phenylethyl benzoate, with methyl benzoate being the most abundant volatile in the group (Negre *et al*., 2003;

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Verdonk *et al*., 2003, 2005; Underwood *et al*., 2005; Verdonk, 2006). Emission of these volatiles is circadian with highest emission levels at night (20.00 h to 02.00 h), while the petal limb is the tissue primarily responsible for their emission (Kolosova *et al*., 2001; Verdonk *et al*., 2003).

The phytohormone ethylene is a volatile molecule necessary for co-ordinating a wide range of physiological processes throughout the plant, including wound responses, herbivore attack, and floral senescence (Abeles *et al*., 1992; Bleecker and Kende, 2000; von Dahl and Baldwin, 2007). In MD flowers, pollination-induced ethylene is synthesized sequentially throughout the flower, ultimately resulting in corolla senescence (Tang and Woodson, 1996; Wilkinson *et al*., 1997; Holden *et al*., 2003; Jones *et al*., 2003). Exposure to both exogenous and pollination-induced ethylene also results in a rapid decrease in emission of all petunia floral volatiles (Underwood *et al*., 2005). This is likely to be caused by a decrease in transcript levels of the biosynthetic genes that are responsible for their biosynthesis as has been shown for *BENZOIC ACID/SALICYLIC ACID CARBOXYL METHYLTRANSFERASE1* and *2* (*PhBSMT1* and *2*) which are responsible for the synthesis of methyl benzoate (Negre *et al*., 2003; Underwood *et al*., 2005).

BENZOYLCOA:BENZYL ALCOHOL/PHENYLETHANOL BENZOYLTRANSFERASE (*PhBPBT*) catalyses the conversion of benzoyl-CoA and benzyl alcohol to benzyl benzoate and also of benzoyl-CoA and 2-phenylethanol to phenylethyl benzoate (Boatright *et al*., 2004). A flower-specific RNAi approach showed that when the transcription of *PhBPBT* is lowered, the direct products benzyl benzoate and phenylethyl benzoate are almost completely absent, while levels of benzyl alcohol and benzaldehyde have increased (Orlova *et al*., 2006). Benzyl benzoate is a volatile produced by several plant species including *Clarkia breweri* (Raguso and Pichersky, 1995), *Gymnadenia conopsea* (Huber *et al*., 2005), *Petunia axillaris* (Hoballah *et al*., 2005), *Dianthus caryophyllus* (Schade *et al*., 2001), and *Petunia* × *hybrida* (Verdonk *et al*., 2003; Boatright *et al*., 2004; Underwood *et al*., 2005). Past *in vitro* experiments have indicated that benzyl benzoate could play a role in pollinator attraction (Shields and Hildebrand, 2000; Hoballah *et al*., 2005; Huber *et al*., 2005; Omura and Honda, 2005) or plant defence (miticide) (Dimri and Sharma, 2004; Harju *et al*., 2004), two common physiological roles attributed to floral volatiles.

To date, research involving PhBPBT has focused predominately on its role in biochemistry and floral volatile biosynthesis in petunia, but a role in vegetative tissue has not been shown. Following mechanical wounding of leaf tissue, transcript levels of *CbBEBT* accumulate rapidly 4–6 h after wounding (D'Auria *et al*., 2002). Additionally, HRS201, encoded by a gene rapidly induced following *Pseudomonas solanacearum* infection in tobacco leaves, has been shown to catalyse the conversion of benzyl alcohol and benzoyl-CoA to benzyl benzoate *in vitro* (Czernic *et al*., 1996). The primary goal of this study was to determine the effects of ethylene on *PhBPBT* transcript levels in the corolla following pollination, and in the leaves following mechanical wounding. Here it is shown that *PhBPBT* is a single copy gene with multiple forms of regulation. Ethylene differentially regulates *PhBPBT* transcript levels in corolla, ovary, and leaf tissue of MD. Additionally, an increase in *PhBPBT* transcript in the leaves resulted in an ethylene-dependent increase in internal benzyl benzoate pools following repeated wounding.

Materials and methods

Plant material

Petunia×*hybrida* cv. MD, 35S::*etr1-1* line 44568 (Wilkinson *et al*., 1997) and *PhBPBT* RNAi transgenic petunias were grown as described previously (Underwood *et al*., 2005).

Selection and identification of PhBPBT

In cDNA libraries (Underwood, 2003) that were selected for cDNAs that were highly expressed in the flower, a 1.7 kb cDNA was identified coding for a 460 amino acid protein (PhBEBT1; Accession no. AAT68601) with 100% homology to BENZOYL-COENZYME A (CoA):BENZYL ALCOHOLPHENYLETHANOL BENZOYLTRANSFERASE (BPBT; Accession no. AAU06226.1) from *Petunia*×*hybrida*, and subsequently named PhBPBT.

Generation of the RNAi silencing construct

Two fragments of *PhBPBT* (295–638 and 295–1139) were amplified via PCR, ligated end-to-end in a sense/antisense orientation, ligated downstream of the FMV constitutive promoter, and transformed into MD as described previously (Dexter *et al*., 2007) Thirty-five primary transformants were recovered and grown to maturity under previously described greenhouse conditions, and plants showing reduced *PhBPBT* expression and benzyl benzoate emission were identified. Flowers from these plants were then self-pollinated and T_1 progeny were grown. Positive lines were then screened for 3:1 segregation of the transgene via PCR (verifying the presence of the *nptII* gene), *PhBPBT* expression, and benzyl benzoate emission. Flowers from PCR-positive plants from lines demonstrating 3:1 segregation were again selfed, the progeny sown, and homozygous lines identified via PCR (*nptII*).

PhBPBT *expression analysis*

Tissue was collected to determine *PhBPBT* expression levels for the following experiments: circadian/light-dependent regulation, *PhBPBT* RNAi plants, ethylene treatment (whole flowers and leaves), post-pollination, and wounding. To test circadian/light-dependent regulation, whole flower tissue was collected at 6 h intervals beginning at 10.00 h in the morning as described previously (Underwood *et al*., 2005). For *PhBPBT* expression analysis in the *BPBT* RNAi transgenic lines versus MD, whole flowers were collected at 20.00 h. For expression after ethylene treatment, MD and 44568 (*etr1-1*) (Wilkinson *et al*., 1997) whole flowers were treated with ethylene and dissected into the petal limb, petal tube, ovary, and stigma/style as described previously (Underwood *et al*. 2005). MD and 44568 leaves were collected at 10.00 h, placed in 1% water agar blocks and treated with ethylene or air as described

previously (Underwood *et al*., 2005). For post-pollination expression analysis, MD and 44568 flowers 1 d before anthesis were either self-pollinated of set aside as a non-pollinated control. Beginning at 10.00 h, both pollinated and non-pollinated flowers from MD and 44568 plants were collected and immediately dissected into petal limb, petal tube, ovary, and stigma/style tissue. This process was then repeated at 12 h intervals up to 60 h after pollination. For leaf wounding experiments, MD and 44568 leaves were wounded by scraping 25% of the adaxial epidermis, starting at the leaf tip and working toward the petiole. Tissue was wounded 0×, 1× (initial wound at 0 h), 2× (0 and 6 h after initial wounding), or 3× (0, 6, and 12 h after initial wounding) times beginning at 10.00 h. Both wounded and unwounded leaves were then excised from the plants at the predetermined time points. In all cases, total RNA was extracted from collected tissues as described previously (Underwood *et al*., 2005). Quantitative RT-PCR using Taqman One-Step RT-PCR reagents (Applied Biosystems, Foster City, CA, USA), the following primers and probe (*PhBPBT* reverse primer: 5′-GAAA-TAAGAAAGGTGAGAATGGGATT-3'; *PhBPBT* forward primer: 5′-AGCTCCTTGACGAATTTTTCCA-3'; *PhBPBT* probe: 5′-/56FAM/TGGTCCCTATATGTTTGCCTGGCTTTGC/ 3BHQ_1/-3'), a series of *in vitro* transcribed *PhBPBT* standards, and 1 µl of 100 ng μl^{-1} RNA were then used to quantify mean *PhBPBT* levels as described previously (Underwood *et al*., 2005). All data with the exception of 24 h and 48 h wounded tissue were then reported as a percentage of total mRNA ±standard error. For 24 h and 48 h wounded tissue, results were reported as a fold-increase in *PhBPBT* transcript levels as compared with respective unwounded controls ±standard error.

Volatile analysis

Emitted volatiles and internal volatile pools were quantified every 6 h for a period of 1 h as described previously (Dexter *et al*., 2007).

Internal benzyl benzoate analysis

To determine internal benzyl benzoate pools following mechanical wounding, MD and 44568 leaf tissue was wounded by scraping 25% of the adaxial epidermis starting at the leaf tip and working toward the petiole. Tissue was wounded 0×, 1× (initial wound at 0 h), 2× (0 and 6 h after initial wounding), or 3× (0, 6, and 12 h after initial wounding) times beginning at 09.00 h. Two leaves were then collected from unwounded and wounded 1×, 2×, and 3× MD and 44568 leaves at 24 h and 48 h after initial wounding. This experiment was repeated twice (three times for 3× 24 h wounded MD and 44568 tissue), resulting in four sets of two leaves (10 sets of two leaves for 3× 24 h MD and 44568 wounded tissue). Pools of volatiles were then extracted and quantified via isobutane chemical–ionization gas chromatography–mass spectrometry as described previously (Schmelz *et al*., 2004). The resulting data were reported as a fold-increase in benzyl benzoate levels over unwounded controls for 1×, 2×, and 3× wounded MD and 44568 tissue ±standard error.

Results

Circadian or light-dependent expression of PhBPBT

Both expression of *PhBPBT* and emission of benzyl benzoate are rhythmic with highest expression at 14.30 h and highest emission 6 h later (20.30 h) (Fig. 1A, B). When the plants were moved to complete darkness, one additional 24 h cycle of expression was observed, followed by a complete loss of rhythmicity (Fig. 1A).

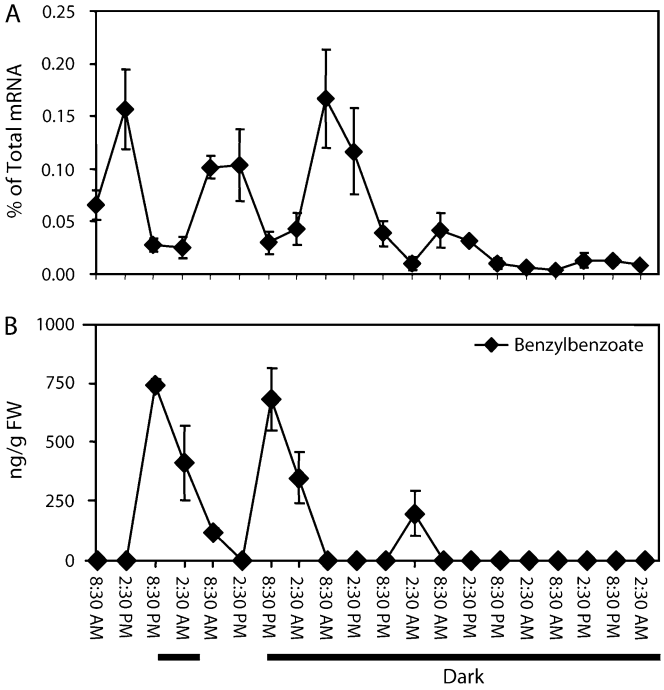

Fig. 1. Transcripts of *PhBPBT* follow a dampened light-dependent circadian trend which is reflected in the emission of benzyl benzoate. (A) Real time (Taqman) *PhBPBT* transcript levels of whole flowers collected at 6 h increments for 5 d. (B) Whole flower emission (±SE) of benzyl benzoate at 6 h increments. Plants were grown in standard greenhouse conditions for 2 d and then transferred to complete darkness.

Additionally, benzyl benzoate emission decreased with reduced emission following the first 24 h cycle in the dark, while no emission was measured thereafter (Fig. 1B).

PhBPBT *RNAi*

To examine the function of *PhBPBT in vivo*, its expression was suppressed through RNAi. Orlova *et al*. (2006) showed a morphological phenotype in lines with reduced expression, reporting larger flowers and leaves, thicker stems, and longer internodes. However, 31 lines with reduced expression and benzyl benzoate emission were produced and examined and the RNAi lines were found morphologically indistinguishable from MD. Three lines with reduced *PhBPBT1* expression (Fig. 2) were selected for further analysis. In these lines the volatile phenotype was dramatically affected. The lines showed an approximate decrease of 90% in benzyl benzoate emission, a 5-fold increase in the direct precursor, benzyl alcohol, a 3-fold increase in benzaldehyde, and a 2-fold increase in 2-phenylethanol. Phenylacetaldehyde, methyl benzoate, and isoeugenol emission levels from both RNAi and MD were not significantly different (Fig. 2). Together these results show that, *in vivo*, *PhBPBT* is critical for the synthesis of benzyl benzoate.

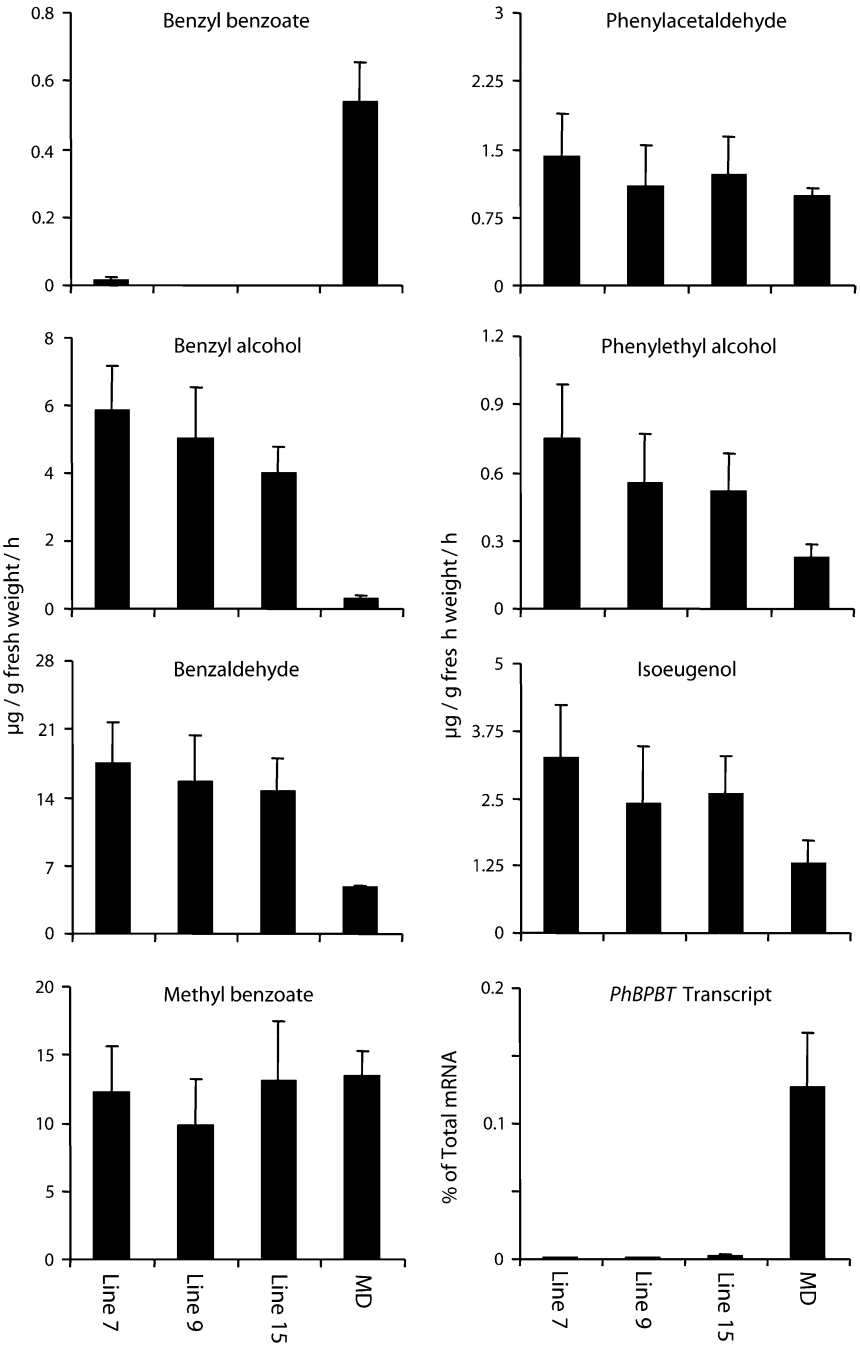

Fig. 2. Reduced emission of benzyl benzoate and increased levels of benzyl alcohol correlate with reduced expression levels of *PhBPBT* are reduced in transgenic *PhBPBT* RNAi lines. Volatiles (±SE) were collected from *PhBPBT* RNAi and MD whole flowers for 1 h at 20.00 h. Three samples were collected from three plants of each line, and averaged to determine average emission. Levels of *PhBPBT* expression (±SE) in RNAi knockdown and wild-type (MD) lines were quantified from whole-flower RNA collected at 20.00 h via Taqman real-time RT-PCR.

PhBPBT *transcript levels following exogenous ethylene treatment*

Benzyl benzoate emission is down-regulated in petunia corollas following exposure to exogenous or pollination-induced ethylene production (Underwood *et al.*, 2005). MD and 44568 petunia flowers were treated with exogenous ethylene and analysed for differences in *PhBPBT* transcript accumulation in separate floral organs (Fig. 3). In MD ovary tissue, exposure to exogenous ethylene resulted in a marginal decrease in transcript at 2 h after treatment compared with 44568 (Fig. 3A). At 10 h after ethylene treatment, *PhBPBT* transcript levels in MD ovary tissue increased, peaking at 24 h (where levels were twice that of 44568), then remained elevated for the

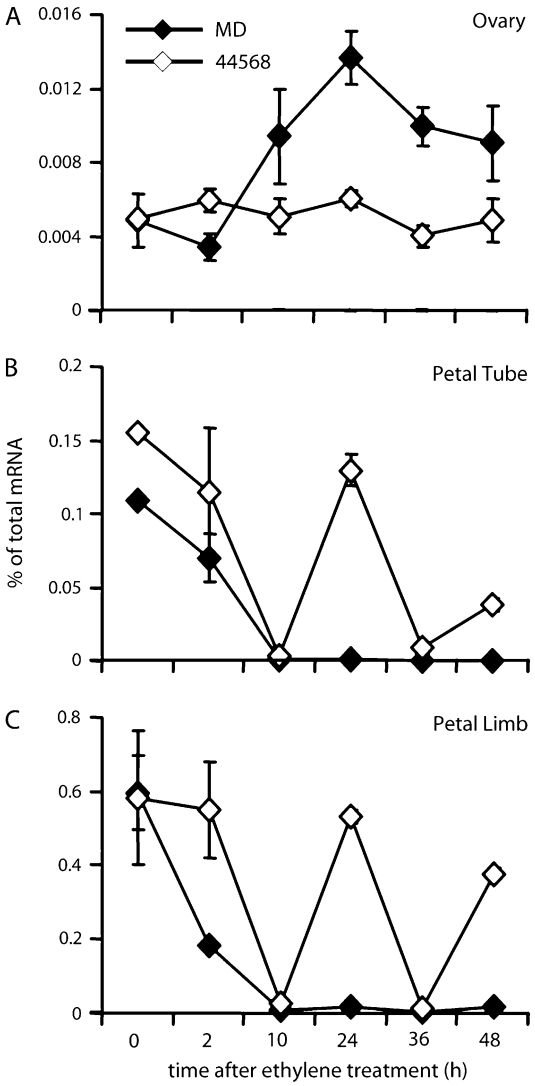

Fig. 3. Ethylene treatment induces *PhBPBT* expression levels in ovary but reduces them in petal tissue. Mean *PhBPBT* transcript levels following treatment with exogenous ethylene. (A) Mean real-time RT-PCR *PhBPBT* transcript levels (n=3, ±SE) were measured in MD (filled diamonds) and line 44568 (open diamonds; *CaMV* 35S::*etr1-1*). (A) Ovary, (B) petal tube, (C) petal limb tissues collected at 2, 10, 24, 36, and 48 h after exogenous ethylene treatment.

remainder of the experiment. In the corolla limb and tube tissue, a marginal decrease in *PhBPBT* transcript levels was observed 2 h after ethylene treatment while they were not detectable at 24 h after ethylene treatment compared with 44568 (Fig. 3B, C).

Post-pollination PhBPBT *expression*

Following the pollination of MD flowers, ethylene is first synthesized from the stigma/style 2–4 h after pollination coinciding with pollen tube growth (Tang and Woodson, 1996; Wilkinson *et al*., 1997; Holden *et al*., 2003; Jones *et al*., 2003). A second period of ethylene synthesis coinciding with fertilization occurs in the stigma/style and ovary 24 h after pollination, followed by ethylene synthesis in the corolla (petal tube and petal limb) from 24 h to 36 h after pollination (Tang and Woodson, 1996; Jones *et al*., 2003). In ovary tissue, *PhBPBT* transcript levels were equivalent through 24 h after pollination among all treatments (Fig. 4A). At 36 h after pollination, both 44568 and MD pollinated ovary tissue contained slightly elevated *PhBPBT* transcript levels compared with their respective non-pollinated controls. By 48–60 h, transcript levels in MD pollinated ovary tissue were 3-fold higher than pollinated 44568 ovary tissue, and 10-fold higher than levels observed in both non-pollinated controls (Fig. 4A). In the petal tube, *PhBPBT* levels were equivalent among all treatments through 12 h after pollination (Fig. 4B). At 24 h after pollination, *PhBPBT* levels in both MD treatments were lower than both 44568 treatments, with MD pollinated transcript levels being slightly higher than MD non-pollinated transcript levels. By 48 h after pollination, *PhBPBT* transcript levels were substantially lower in MD pollinated petal tube tissue compared with the other three treatments. Prior to pollination, *PhBPBT* transcript abundance in the petal limb tissue was >75-fold higher than levels measured in ovary and petal tube tissue (Fig. 4). After pollination, petal limb transcript levels remained equivalent among all treatments through 36 h after pollination (Fig. 4). At 48 h after pollination, a 2-fold decrease in *PhBPBT* was observed in MD pollinated petal limb tissue compared with the 44568 and both non-pollinated controls. However, *PhBPBT* transcript levels were still 10-fold higher than transcript levels quantified in MD pollinated ovary and 44568 pollinated and non-pollinated petal tube tissue. This coincides with the previous observation of decreased benzyl benzoate emission 36–48 h after pollination (Underwood *et al*., 2005).

PhBPBT transcript levels in the ovary were measured during a 5 d period after pollination to investigate if increased transcript levels in ovary tissue continued beyond the range of the original time-course (60 h). The effects of ethylene on *PhBPBT* transcript levels were transient, with peak transcript levels measured 2–3 d after pollination in MD ovaries compared with 44568 and non-pollinated controls (Fig. 4D). By 4–5 d after pollination, *PhBPBT* transcript levels in pollinated MD ovary tissue were no different from those measured in the other three treatments. To determine if this increase in *PhBPBT* transcript resulted in increased benzyl benzoate biosynthesis, internal volatiles were extracted from MD ovary tissue collected at 3 d and 6 d after pollination. However, benzyl benzoate pools in these extracts were below detection limits (data not shown).

PhBPBT *transcript levels in petunia leaves treated with ethylene*

Two hours after exogenous ethylene or air treatment of excised 44568 and MD leaves, an induction of *PhBPBT*

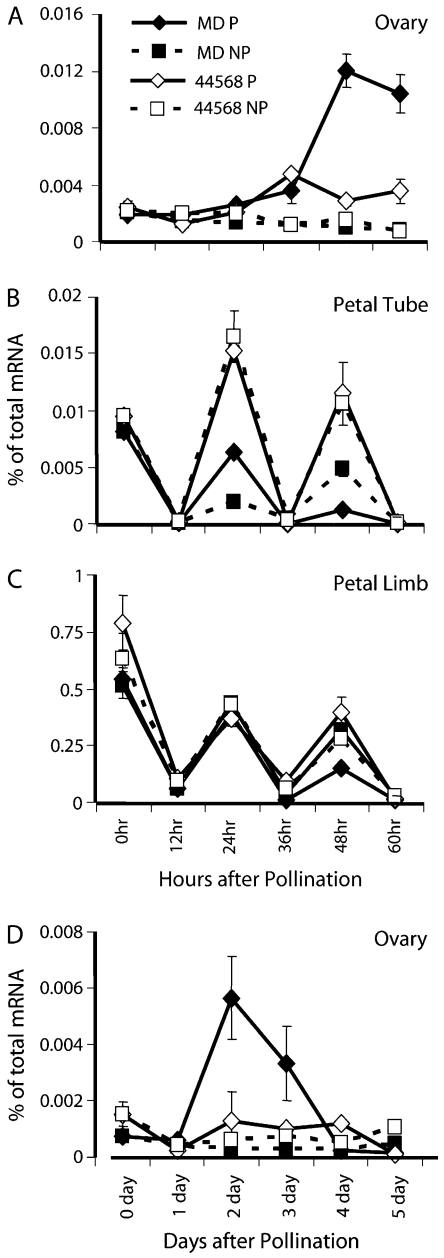

Fig. 4. The effect of pollination-induced ethylene on *PhBPBT* transcript and internal benzyl benzoate levels. (A) *PhBPBT* transcript levels (n=3, ±SE) in pollinated and non-pollinated MD and 44568 (CaMV 35S::etr1-1) ovary tissue. (B) *PhBPBT* transcript levels (n=3, ±SE) in pollinated and non-pollinated MD and 44568 petal tube tissue. (C) *PhBPBT* transcript levels (n=3, ±SE) in pollinated and non-pollinated MD and 44568 petal limb tissue. (D) Mean *PhBPBT* transcript levels (n=3, ±SE) in both MD and 44568 pollinated or non-pollinated ovary tissue.

transcript levels was observed in MD leaves treated with ethylene compared with all other treatments (Fig. 5). By 6 h after treatment, *PhBPBT* levels further increased in ethylene-treated MD leaves compared with 44568 leaves, with peak transcript levels comparable to peak levels observed in MD flower tissue. Therefore, unlike in the corolla tissue but similar to that in ovary tissue, treatment

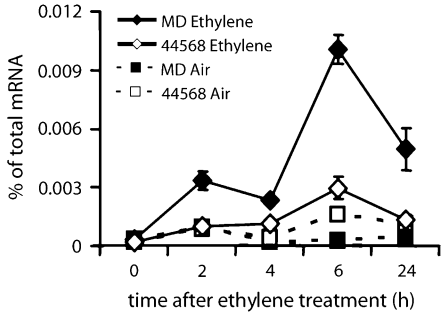

Fig. 5. Induction of transcript levels in leaf tissue is ethylene dependent. Mean *PhBPBT* transcript levels (n=3, ±SE) in petunia leaf tissue following exogenous ethylene treatment. Total RNA was extracted from MD and 44568 (CaMV 35S::etr1-1) leaf tissue following treatment with exogenous ethylene or air, and analysed via Tagman real-time RT-PCR.

with ethylene results in increased levels of *PhBPBT* in the leaves.

Ethylene-dependent regulation of PhBPBT *transcript levels following multiple wounding events*

Petunia leaves were wounded three times at 6 h intervals (0, 6, and 12 h after initial wounding) to determine the effect of wound-induced ethylene on *PhBPBT* transcript levels (Fig. 6A). During the period after the first wounding event (0–6 h after initial wounding), no change in *PhBPBT* transcript levels was observed in MD or 44568 wounded leaves compared with unwounded controls (Fig. 6B). After the second wounding event, *PhBPBT* transcript levels in 44568 wounded leaf tissue were slightly elevated compared with the other three treatments. By 12 h after wounding, *PhBPBT* transcript levels in both MD and 44568 wounded leaf tissues were elevated compared with unwounded controls. Following a third wounding event, *PhBPBT* transcript levels in both controls and wounded MD leaves remained static, while levels in wounded 44568 leaves rapidly increased (Fig. 6B, C). After 48 h, levels of *PhBPBT* transcript in both MD and 44568 had almost returned to their original level. Levels of benzyl benzoate were slightly increased in both MD and 44568 wounded tissue compared with their respective controls (Fig. 6D). In MD leaf tissue wounded 2× or 3× times, internal benzyl benzoate pools were elevated compared with 1× and unwounded MD treatments. In 44568 wounded leaf tissue, no significant increase in benzyl benzoate pools was observed following 2× wounding events; however, following a third wounding event, a substantial increase in benzyl benzoate was observed over 44568 leaf tissue wounded 1× and 2× (Fig. 6D). While a >50-fold increase in *PhBPBT* transcript was observed in 44568 3× wounded tissue compared with MD 3× wounded leaf tissue (Fig. 6C), there was little difference in internal benzyl benzoate levels in MD or 44568 wounded leaf tissue (Fig. 6D). These results indicate that in MD

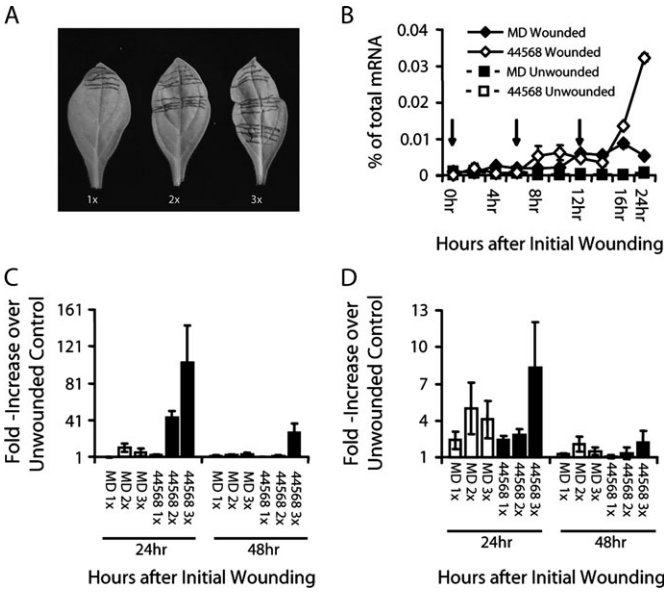

Fig. 6. *PhBPBT* transcript and internal volatile levels in petunia leaves following repeated wounding events. (A) MD petunia leaves wounded 1×, 2×, and 3× times. (B) Wounded and unwounded MD and 44568 (CaMV 35s::etr1-1) leaves were collected at 2 h increments for 16 h plus an additional 24 h collection. Tissue was wounded three times (0, 6, 12 h indicated by black arrows). Mean *PhBPBT* transcript levels (n=3, ±SE) were quantified via real-time RT-PCR. (C) Fold increases in *PhBPBT* transcript (n=3, ±SE) in MD and 44568 wounded leaves as compared with respective unwounded controls. Leaves were wounded 1× (0 h), 2× (0, 6 h), or 3× (0, 6, 12 h) times, and total RNA was extracted at 24 h and 48 h after initial wounding. (D) Fold increases in benzyl benzoate levels (n=4, ±SE) in MD and 44568 wounded leaves as compared with respective unwounded controls. Leaves were wounded 1× (0 h), 2× (0, 6 h), or 3× (0, 6, 12 h) times and volatiles were extracted at 24 h and 48 h after initial wounding.

leaves *PhBPBT* transcript and benzyl benzoate levels are transiently elevated in response to mechanical wounding yet transcripts do not result in quantitative relationships with product pools, suggesting additional levels of regulation.

Discussion

Circadian expression of PhBPBT *is light dependent*

All genes involved in floral benzenoid biosynthesis that have been characterized in petunia (*PhBSMT1* and *2*, *PhBPBT*, *PhCFAT*, *PhIGS1*, *PhPAAS*, and *PhODO1*) follow a rhythm, with a maximum expression preceding the nocturnal peak of scent emission by ~2–4 h (Negre *et al*., 2003; Boatright *et al*., 2004; Underwood *et al*., 2005; Verdonk *et al*., 2005; Kaminaga *et al*., 2006; Koeduka *et al*., 2006; Orlova *et al*., 2006; Dexter *et al*., 2007). Here it is shown that, because the rhythm is dampened after the plants have been placed in complete darkness, the regulation of *PhBPBT* transcription is both light dependent and circadian in origin. A similar pattern of expression has previously been described for *PhBSMT*; when moved into complete darkness, *PhBSMT* transcript abundance was no longer rhythmic, with a total loss of rhythmicity observed after 1 d in constant dark conditions (Underwood *et al*., 2005).

PhBPBT *RNAi lines show altered volatile production but no morphological phenotype*

A decrease in PhBPBT function results in an accumulation of its substrates, benzyl alcohol and 2-phenylethanol (Fig. 2), as was seen before in MD plants with a flower-specific RNAi approach (Orlova *et al*., 2006). Acetyl-CoA is also a possible substrate for PhBPBT (Boatright *et al*., 2004), and when this is used instead of benzoyl-CoA, benzyl acetate and 2-phenylethyl acetate are formed. These substances were present at levels below the limits of detection yet we speculate that the trace pools present may be further reduced. Benzyl alcohol is not only converted to benzyl benzoate but also reversibly to benzaldehyde and to methyl benzoate in petunia petal tissue (Boatright *et al*., 2004). In agreement with this, a 3-fold accumulation of benzaldehyde is observed in RNAi plants as compared with wild type (Fig. 2), but no change in methyl benzoate emission or internal accumulation was observed.

Flower-specific silencing of the *PhBPBT* gene has been reported to affect the morphology of MD plants dramatically (Orlova *et al*., 2006). In short, Orlova *et al*. (2006) reported larger flowers, longer internodes, larger leaves, and thicker stems. The 31 RNAi lines created in this study using the constitutive FMV promoter (Richins *et al*., 1987) were morphologically indistinguishable from MD plants (RJ Dexter, JC Verdonk, DG Clark, personal observation). The phenotype described by Orlova *et al*. (2006) was similar to a polyploid phenotype that is routinely observed when MD plants come out of tissue culture. These polyploid plants produce large flowers, with larger organs that are delayed in blooming, while the plants have significantly thicker stems and large leaves. To investigate this the RNAi lines were requested from Dr Natalia Dudareva's group and the amount of genetic material determined by flow cytometry. These plants showed a higher content of genetic material (data not shown), so subsequently the number of amyloplasts of stomatal guard cells in epidermal peels was analysed (Qin and Rotino, 1995). It was clear that the number of amyloplasts in the stomatal guard cells of these transgenic lines was doubled as compared with MD, and therefore we suggest that the morphological phenotype described in Orlova *et al*. (2006) is caused by a ploidy change rather than the silencing of the *PhBPBT* gene.

Ethylene differentially regulates PhBPBT *transcription in petunia floral tissue*

Both pollination and exogenous ethylene treatment cause a down-regulation of *PhBSMT* and *PhCFAT* transcript

levels (Negre *et al*., 2003; Underwood *et al*., 2005; Dexter *et al*., 2007), resulting in a decrease of methyl benzoate and isoeugenol emission. The same treatment also causes a decrease in benzyl benzoate emission (Underwood *et al*., 2005). While ethylene treatment causes a down-regulation of floral *PhBPBT* expression (Fig. 4), transcript levels in 44568 and MD pollinated and non-pollinated ovary and corolla tissue remained equivalent, unaffected by the initial period (<24 h after pollination) of rapid ethylene synthesis by the stigma/style (Jones *et al*., 2003). Following successful fertilization (>24 h after pollination), ethylene synthesized both in the ovary and corolla then differentially regulates *PhBPBT* transcript levels, resulting in an increase of transcript levels in the ovary and decreased transcript levels in the corolla (Fig. 4). The down-regulation of *PhBPBT* in the corolla does not coincide with the decrease in benzyl benzoate emission (Fig. 1); therefore, this decrease is likely to be caused by an absence in substrates, probably caused by down-regulation of genes upstream in the pathway, like *PHENYLALANINE AMMONIA LYASE*, *5-ENOL- PYRUVYL SHIKIMATE-3-PHOSPHATE SYNTHASE*, or *ODORANT1* after ethylene treatment.

Ethylene-dependent regulation of PhBPBT *transcript and benzyl benzoate biosynthesis in petunia vegetative tissue after wounding*

In the flower, *PhBPBT* transcript levels and subsequent benzyl benzoate biosynthesis are down-regulated by ethylene. Because in *Clarkia* wounding was shown to up-regulate *CbBEBT* expression levels in leaves (D'Auria *et al*., 2002) and because ethylene was shown to be synthesized in *Petunia×hybrida* leaves following mechanical wounding (Gomez, 1996; Boatright, 2000), it is possible that this ethylene is necessary for the up-regulation of *PhBPBT* transcript levels in leaves after wounding. Treatment of petunia leaves with exogenous ethylene resulted in increased levels of *PhBPBT* transcript in MD leaves but not 44568, initially indicating that ethylene could up-regulate *PhBPBT* transcription in the leaves (Fig. 5). Interestingly, while exogenous ethylene treatment resulted in increased *PhBPBT* transcript levels in MD tissue compared with 44568, transcript levels in 44568 leaf tissue wounded 2× or 3× times were substantially (>50-fold) higher than MD (Fig. 6). This suggests that ethylene instead plays an inhibitory role in the regulation of *PhBPBT* transcript levels following wounding in leaves. Because there was no difference between MD and 44568 benzyl benzoate levels, while wounding of 44568 leaves resulted in a substantial increase in transcript compared with MD it is likely that the benzyl benzoate levels are regulated by the availability of their precursors rather than by a signal dependent on ethylene.

Possible physiological role of benzyl benzoate in petunia

While little is known about the physiological role of benzyl benzoate *in vivo*, some *in vitro* research has provided evidence to support a role for benzyl benzoate in either pollinator attraction or plant defence. Benzyl benzoate is a volatile, with little perceived odour to humans, that has been isolated from many plant species including petunia MD (Knudsen *et al*., 1993; Verdonk *et al*., 2003). For moth pollinators, however, it is likely that benzyl benzoate is attractive, because exposure to benzyl benzoate excites receptor cells in the antenna of *Mythimna conigera* (Huber *et al*., 2005) and *Manduca sexta* (Shields and Hildebrand, 2000; Hoballah *et al*., 2005). By contrast, benzyl benzoate has also been reported to be a repellent involved in defence; it is an effective treatment against common mites such as *Tyrophagus putrescentiae* (Schrank) and sheep mange (Dimri and Sharma, 2004; Harju *et al*., 2004).

It is possible that PhBPBT and subsequent benzyl benzoate production play a physiological role in the protection of the ovary tissue from pathogens immediately following senescence of the corolla tissue (days 2–3), a potentially vulnerable time in ovary development (Fig. 4D). The wound-induced induction of *PhBPBT* transcript levels and benzyl benzoate pools is indicative of a defence role for benzyl benzoate within the leaves. While a physiological role for *PhBPBT* and subsequent benzyl benzoate in petunia leaf tissue (after repeated wounding) cannot be ruled out; even at its highest levels, *PhBPBT* transcript levels within MD and 44568 leaf tissue are substantially lower than those observed in the petal limb (Fig. 4D). The relatively lower levels of both the wound- and fertilization-induced *PhBPBT* transcription in the leaves and ovary (both long-term regenerative tissues, while petals are terminal) as compared with the petals suggests that the primary function of *PhBPBT* and subsequent benzyl benzoate is in the petal limb prior to fertilization. The availability of the present *PhBPBT* RNAi lines will be ideal in determining the importance of PhBPBT and subsequent benzyl benzoate production in defence against pathogens.

In conclusion, it has been shown that *PhBPBT* is differentially regulated in response to ethylene perception, depending on the plant organ. The specificity of this expression pattern is consistent with the hypothesis that *PhBPBT* and subsequent benzyl benzoate production is involved in both pollinator attraction and also defence against biotic attack. Diploid transgenic plants created in this work that exhibit modified benzyl benzoate production will be invaluable in future studies aimed at identifying further the physiological and multitrophic roles of this floral benzenoid.

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